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The Relations of Genus *Glycine* Subgenus Soja and *Glycine formosana* Hosok. Collected from Taiwan: Revealed by RAPD Analysis

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Glycine gracilis Skv., *G. max* (L.) Merr., *G. soja* Siebold & Zucc., and *G. formosana* Hosok. were analyzed in order to clarify their inter-species relations by random amplified polymorphic DNA (RAPD). The results showed that *G. gracilis* and *G. max* are closest among the four species, and that *G. formosana* from Taiwan is least related to other ones. They can be classified into the following three groups: A) *G. max* and *G. gracilis*, B) *G. soja*, and C) *G. formosana*. From these results and previous reports (Thseng et al. 1999), we consider that the Taiwanese wild soja plant is distinct, and the name *G. formosana* Hosok. is appropriate for the plant.

Key words: *Glycine* Subgenus Soja, *Glycine formosana*, RAPD analysis

Introduction

Glycine formosana Hosok. is distributed over the grassland along the riverside and roadside in northern Taiwan, at Guanshi, Herngshan, and Dahshi. It is a twining annual herb, with lanceolate leaflets, and small, dark-brown, ellipsoidal seeds. Its chromosome number is 2n=40. In taxonomy, *G. formosana* had once been considered as the same species as *G. soja* (Huang and Ohashi 1977), *G. max* ssp. *soja* (Huang and Huang 1987), or *G. max* ssp. *formosana* (Hosok.) Tateishi & H. Ohashi (Tateishi and Ohashi 1992). The cross compatibility between *G. formosana* and *G. max* is normal (Tang and Chen 1959). *Glycine soja* is mainly distributed in China, Korea, Japan, and Russia, and is considered as the ancestor of *G. max* (Ahmad et al. 1976, Hadley and Hymowitz 1973, Hymowitz and Singh 1987). *Glycine gracilis* Skv. is also one of the member of *G. max*. It might be an evolutionary intermediate between *G. soja* and *G. max* or a natural hybrid between *G. soja* and *G. max* (Hadley and Hymowitz 1973, Sisson et al. 1978, Broich and Palmer 1981, Hui et al. 1995). Thseng et al. (1999) reported that *G. formosana* showed a significant difference from *G. max* and *G. soja* on RAPD profile. In this experiment, the relationship among *G. formosana*, *G. soja*, *G. max*, and *G. gracilis* from the RAPD markers was investigated.

Materials and Methods

DNA extraction

Thirteen accessions of *Glycine max* were collected from Taiwan, USA, and China. Two accessions of *G. gracilis* were collected from China. Three accessions of *G. formosana* were collected from Taiwan. Nine accessions of *G. soja* were collected from China, Korea and Japan (Table 1). Twenty seeds of each accession were grown to the flowering stage in a controlled environment. DNA was extracted using a modified version of Doyle et al. (1990). Leaf

materials (1 g fresh weight) were ground to fine powder in liquid nitrogen. The powdered leaf tissue was transferred to a beaker, and 5 ml of pre-heated extraction buffer (2 % CTAB; 1.4 M NaCl, 0.2 % 2-mercraptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH=8.0) was added. After 30 minutes at 60°C, 5 ml of Chloroform-isoamyl alcohol (24:1 v/v) was added, and the upper clear part of the solution was collected by centrifugation at 10000 xg for 5 minutes at 4°C. Then, 5 ml of Chloroform-isoamly alcohol was again added, and the cell debris was removed by another 10 minutes of centrifugation at 4°C. The DNA was precipitated by the addition of 3.3 ml of isopropanol and recovered by centrifugation for 5 minutes at 10000 xg after incubation in a freezer (−20 °C) for 3 hours. The pellet was dried and redissolved in 2 ml TE buffer (10 mM Tris-

Table 1. Accession number, taxon and origin of genus *Glycine* subgenus Soja used in the experiment

Acc. no.	Race	Taxon	Origin	Preservation institution
1	Taipei wutou (L)	*Glycine max*	Taiwan	Dept. of Plant Germplasm, Taiwan Agriculture Institute
2	Chinjen wutou (L)	*Glycine max*	Taiwan	〃
3	Ilan wutou (L)	*Glycine max*	Taiwan	〃
4	Hernchuen wutou (L)	*Glycine max*	Taiwan	〃
5	Pingtong chinpitou (L)	*Glycine max*	Taiwan	〃
6	Hsinying tatou (L)	*Glycine max*	Taiwan	〃
7	Taiwan wutou (L)	*Glycine max*	Taiwan	〃
8	Brown tou (L)	*Glycine max*	Taiwan	〃
9	Medium gree (C)	*Glycine max*	USA	〃
10	Minsoy (C)	*Glycine max*	USA	〃
11	Peking (C)	*Glycine max*	USA	〃
12	Tongjea 1 (C)	*Glycine max*	China	〃
13	Wenhon 5 (C)	*Glycine max*	China	〃
14	Wild soybean	*Glycine gracilis*	China	Dept. of Agronomy, Chung Hsing University
15	Wild soybean	*Glycine gracilis*	China	〃
16	Wild soybean	*Glycine formosana*	Taiwan	〃
17	Wild soybean	*Glycine formosana*	Taiwan	〃
18	Wild soybean	*Glycine formosana*	Taiwan	〃
19	Wild soybean	*Glycine soja*	China	Lab. of Plant Genetic and Evol., Hokkaido University
20	Wild soybean	*Glycine soja*	China	〃
21	Wild soybean	*Glycine soja*	China	〃
22	Wild soybean	*Glycine soja*	China	〃
23	Wild soybean	*Glycine soja*	Korea	〃
24	Wild soybean	*Glycine soja*	Korea	〃
25	Wild soybean	*Glycine soja*	Korea	〃
26	Wild soybean	*Glycine soja*	Japan	〃
27	Wild soybean	*Glycine soja*	Japan	〃

(L): local variety, (C): cultivar.

HCl, 1 mM EDTA, pH=7.4), 0.2 ml 2M NaCl, and 5 ml alcohol (95 %) 1 hour at −20°C. Then the precipitate was collected by centrifugation at 10000 xg for 5 minutes at 4°C. The pellet was re-dissolved in 5 ml alcohol (95 %) and re-collected by centrifugation at 12000 xg for 5 minutes at 4°C. The final pellet was dried and dissolved in 0.5 ml TE buffer. The DNA concentration was determined using a fluorometer and following the procedures supplied by the manufacturer. The extracted DNA was stored at 4°C in a cooler.

DNA amplification

A set of 30 random 10-mer primers (Operon Technologies Inc., USA) were used in single-primed PCR reactions to generate polymorphisms. A 25 μl reaction was set up as follows: 0.2 mM dNTPs, 0.2 uM, 3 $ng\mu l^{-1}$ Genomic DNA and 0.04 $U\mu l^{-1}$ template DNA in 1X buffer. The reaction was overlaid with mineral oil and the reaction mix heated to 94°C for 5 minutes to denature the template DNA. Amplification proceeded on a DNA Thermalcycler (Perkin-Elmer) for 43 cycles of 1 minute at 94°C, 2 minutes at 42°C and 3 minutes at 72°C and a final step of 10 minutes at 72°C. The total volume of the reaction was loaded onto 1.5 % agarose gel containing 0.3 μgml^{-1} ethidium bromide in 0.5X TBE buffer (pH=8.3, with 45 mM Tris-base, 45 mM boric acid and 1 mM EDTA). The samples were separated by electrophoresis at 110 V for 3.5 hours, then the gels were photographed under UV.

Data analyses

Reproducible DNA bands were scored for presence (1) or absence (0) of amplification products. Pair-wise comparisons of sample were used to estimate Jaccard similarity coefficients: a/(a+b+c), where a=band number of positive coincidences in OTUi and OTUj ($i \neq j$; i, j were accessions cords), b=band number of positive incidence only in OTUi, c=band number of positive incidence only in OTUj (Dunn and Everitt 1982). Using SAS

Table 2. The total number of amplified DNA fragment (band) and the numbers of polymorphic band for each primer used in RAPD analysis of genus *Glycine* subgenus Soja (*G. formosana*, *G. soja*, *G. gracilis* and *G. max*)

Operon Primers	Sequence (5' to 3')	Total bands[a]	Operon Primers	Sequence (5' to 3')	Total bands[a]
OPA–11	CAATCGCCGT	8 (8)	OPB–06	TGCTCTGCCC	10 (8)
OPA–12	TCGGCGATAG	11(10)	OPB–07	GGTGACGCAG	8 (6)
OPA–13	CAGCACCCAC	3 (1)	OPB–08	GTCCACACGG	10 (9)
OPA–14	TCTGTGCTGG	7 (7)	OPB–09	TGGGGGACTC	6 (6)
OPA–15	TTCCGAACCC	7 (6)	OPB–10	CTGCTGGGAC	11 (7)
OPA–16	AGCCAGCGAA	8 (7)	OPB–11	GTAGACCCGT	8 (7)
OPA–17	GACCGCTTGT	3 (1)	OPB–12	CCTTGACGCA	7 (6)
OPA–18	AGGTGACCGT	5 (3)	OPB–13	TTCCCCCGCT	6 (5)
OPA–19	CAAACGTCGG	9 (7)	OPB–14	TCCGCTCTGG	9 (7)
OPA–20	GTTGCGATCC	7 (4)	OPB–15	GGAGGGTGTT	5 (4)
OPB–01	GTTTCGCTCC	8 (5)	OPB–16	TTTGCCCGGA	10(10)
OPB–02	TGATCCCTGG	6 (5)	OPB–17	AGGGAACGAG	9 (8)
OPB–03	CATCCCCCTG	6 (4)	OPB–18	CCACAGCAGT	9 (7)
OPB–04	GGATCGGATG	10 (6)	OPB–19	ACCCCCGAAG	13(11)
OPB–05	TGCGCCCTTC	9 (7)	OPB–20	GGACCCTTAC	7 (4)
Total					235(186)

[a] Number within the parenthesis indicates polymorphic band.

Table 3. Similarity index between each pair of 27 accessions of genus *Glycine* subgenus Soja

Accessions	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 *G. max*	0.81	0.92	0.88	0.88	0.82	0.87	0.77	0.85	0.82	0.84	0.77	0.84	0.75	0.74	0.62	0.63	0.63	0.75	0.66	0.72	0.67	0.68	0.74	0.69	0.68	0.69
2 *G. max*		0.85	0.82	0.81	0.82	0.84	0.79	0.82	0.78	0.78	0.81	0.84	0.71	0.71	0.63	0.62	0.62	0.70	0.64	0.71	0.63	0.70	0.70	0.65	0.70	0.66
3 *G. max*			0.86	0.88	0.84	0.89	0.79	0.87	0.82	0.82	0.79	0.84	0.76	0.74	0.65	0.66	0.66	0.75	0.64	0.74	0.68	0.71	0.75	0.71	0.74	0.70
4 *G. max*				0.85	0.80	0.87	0.74	0.83	0.85	0.84	0.78	0.83	0.79	0.77	0.62	0.62	0.63	0.75	0.72	0.72	0.71	0.69	0.75	0.70	0.71	0.69
5 *G. max*					0.85	0.92	0.78	0.83	0.82	0.84	0.73	0.83	0.78	0.75	0.60	0.61	0.61	0.76	0.68	0.72	0.66	0.69	0.75	0.71	0.72	0.69
6 *G. max*						0.83	0.85	0.85	0.80	0.84	0.74	0.78	0.76	0.77	0.66	0.66	0.66	0.70	0.65	0.68	0.63	0.67	0.74	0.69	0.73	0.70
7 *G. max*							0.78	0.85	0.81	0.81	0.74	0.83	0.78	0.76	0.62	0.63	0.63	0.75	0.66	0.73	0.65	0.69	0.73	0.68	0.71	0.69
8 *G. max*								0.82	0.76	0.76	0.74	0.78	0.71	0.72	0.63	0.62	0.63	0.65	0.59	0.65	0.60	0.66	0.71	0.66	0.72	0.64
9 *G. max*									0.85	0.84	0.75	0.78	0.77	0.78	0.62	0.62	0.63	0.74	0.69	0.73	0.67	0.70	0.74	0.71	0.72	0.69
10 *G. max*										0.85	0.73	0.79	0.82	0.82	0.64	0.64	0.64	0.75	0.71	0.73	0.71	0.72	0.75	0.71	0.72	0.67
11 *G. max*											0.71	0.78	0.82	0.81	0.59	0.60	0.60	0.75	0.72	0.71	0.69	0.68	0.73	0.73	0.70	0.70
12 *G. max*												0.83	0.65	0.65	0.65	0.63	0.64	0.66	0.60	0.65	0.61	0.67	0.67	0.65	0.71	0.65
13 *G. max*													0.74	0.74	0.62	0.63	0.63	0.70	0.66	0.71	0.67	0.70	0.73	0.68	0.71	0.65
14 *G. gracilis*														0.93	0.57	0.57	0.58	0.77	0.74	0.74	0.71	0.70	0.72	0.71	0.70	0.68
15 *G. gracilis*															0.57	0.57	0.57	0.74	0.76	0.73	0.73	0.70	0.70	0.71	0.68	0.68
16 *G. formosana*																0.95	0.94	0.61	0.57	0.59	0.55	0.61	0.66	0.59	0.65	0.60
17 *G. formosana*																	0.99	0.61	0.57	0.58	0.55	0.62	0.67	0.60	0.64	0.60
18 *G. formosana*																		0.61	0.57	0.58	0.55	0.62	0.68	0.60	0.65	0.60
19 *G. soja*																			0.79	0.81	0.75	0.74	0.76	0.77	0.70	0.77
20 *G. soja*																				0.75	0.77	0.73	0.69	0.72	0.67	0.72
21 *G. soja*																					0.79	0.71	0.75	0.72	0.72	0.73
22 *G. soja*																						0.68	0.72	0.69	0.66	0.71
23 *G. soja*																							0.73	0.76	0.73	0.71
24 *G. soja*																								0.77	0.76	0.72
25 *G. soja*																									0.72	0.70
26 *G. soja*																										0.75

computer program, the similarity coefficients were used to construct a dendrogram by UPGMA (unweighted pair-group method with arithmetic mean).

Results and Discussion

Thirty primers were used in the RAPD analysis performed on 27 accessions of *Glycine formosana*, *G. soja*, *G. gracilis*, and *G. max*. In 235 bands produced, 186 bands showed polymorphism (79 %) (Table 2). Their similarity coefficients are between 0.43 to 0.99 (Table 3). The results of cluster analysis were shown in Fig. 1, 27 accessions can be divided into three groups.

Group A includes a total of 15 accessions, of which 13 being *G. max* and the other 2 being *G. gracilis*. The similarity coefficients among this group are between 0.71 to 0.92. And two accessions of *G. gracilis* have high similarity coefficient of 0.93. Figure 2 shows

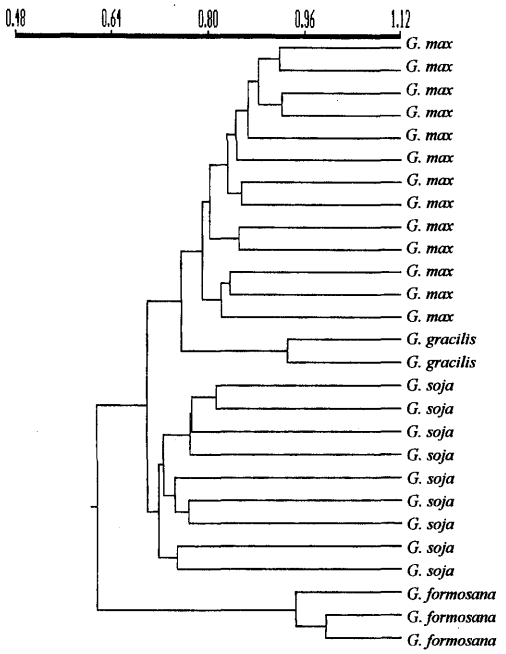

Fig. 1. Dendrogram of genus *Glycine* subgenus Soja based on Jaccard's genetic similarity coefficients by using UPGMA method.

the results of RAPD analysis on OPB-04 primer of all 27 accessions, and 7 bands are produced. *Glycine gracilis* is different from others in that two of its accessions are capable of producing a characteristic band, which is larger than 2 kbp. Figure 3 is another example of *G. gracilis*'s characteristic band. The result of RAPD analysis on OPB-05 primer shows that only the two accessions of *G. gracilis* have a unique band larger than 2 kbp.

Group B includes 9 accessions, all being *G. soja*. The similarity coefficients among this group are between 0.66 to 0.81. However, no unique band of *G. soja* is found on 30 primers after analysis.

Group C includes 3 accessions of *G. formosana*. The similarity coefficients among this group are between 0.94 to 0.99, which are very high. Figure 4 is the result of RAPD analysis on OPB-11 primer. Three accessions of *G. formosana* have a characteristic band of 900 bp, which does not appear in other accessions. Figure 5 is another example of *G. formosana*'s characteristic band. The result of RAPD analysis on OPB-12 primer shows that only the three accessions of *G. formosana* show a characteristic band of 600 bp, being different from other accessions. After several tests, it is found that these two characteristic bands of *G. formosana* are highly repetitive. They could be used as the marker to identify *G. formosana*. Except for these characteristic bands, *G. formosana* is also different from other accessions in that it lacks certain bands or lack in color in certain bands. Figure 6 is a result of RAPD analysis on OPA-15 primer, and it shows that all accessions except *G. formosana* show an evident 700 bp band.

Using gene sequence to investigate the genetic relationship among *G. max*, *G. gracilis*, and *G. soja*, it was found that a natural hybrid or an evolutionary intermediate of *G. gracilis* was between *G. max* and *G. soja*

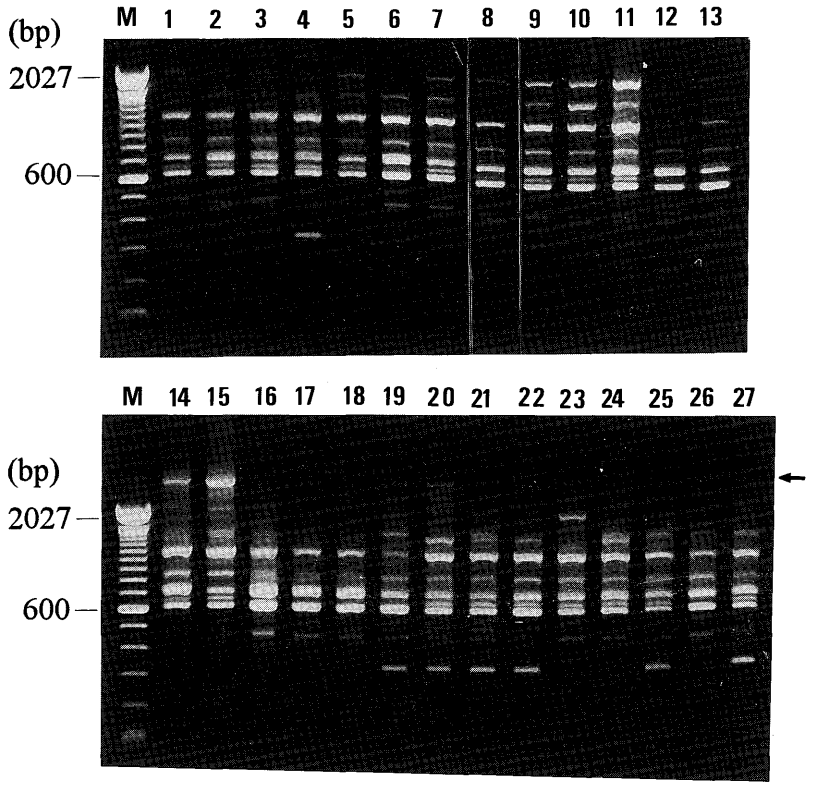

Fig. 2. RAPD profiles from 27 accessions of genus *Glycine* subgenus Soja using primer OPB–04. M: DNA marker; Acc. no.: *G. max* (1–13), *G. gracilis* (14–15), *G. formosana* (16–18), *G. soja* (19–27).

(Broich and Palmer 1981, Close et al. 1989, Hui et al. 1996). The research on DNA sequences classified *G. gracilis* and *G. soja* as the same group (Terry et al. 1995). Furthermore, on the research about the cpDNA of soybeans, Sisson et al. (1978) found that part of the *G. gracilis* and the old cultivated soybeans (*G. max*) were indistinguishable. In this experiment, the results indicated that the diversity of intra-species was not large in the accessions of *G. gracilis*'s, and it's the *G. gracilis* was grouped with *G. max*. Hence, we can consider *G. gracilis* as a variety of *G. max* and not a member of subgenus Soja.

It had been reported that *G. formosana* in Taiwan was different from the *G. soja* in Japan, Korea, and China in seed sizes and pod lengths (Thseng et al. 1999). In the variation of allozymes, *G. formosana* had a distinctive *LAP1-d* band to separate itself with other *G. soja* (Thseng et al. 1999). Same results were obtained in this experiment that characteristic bands of *G. formosana* were found by RAPD analysis on 30 random primers. These bands could be used as classification markers. After the similarity coefficient calculation and cluster analysis, high intra-species similarity was found, and the difference from other *G. soja* is distinct. In the relation between *G. formosana* and *G. max*, the similarity coefficients between *G. formosana* and *G. max* are between 0.59 to 0.66. Hsing et al. (1995) had

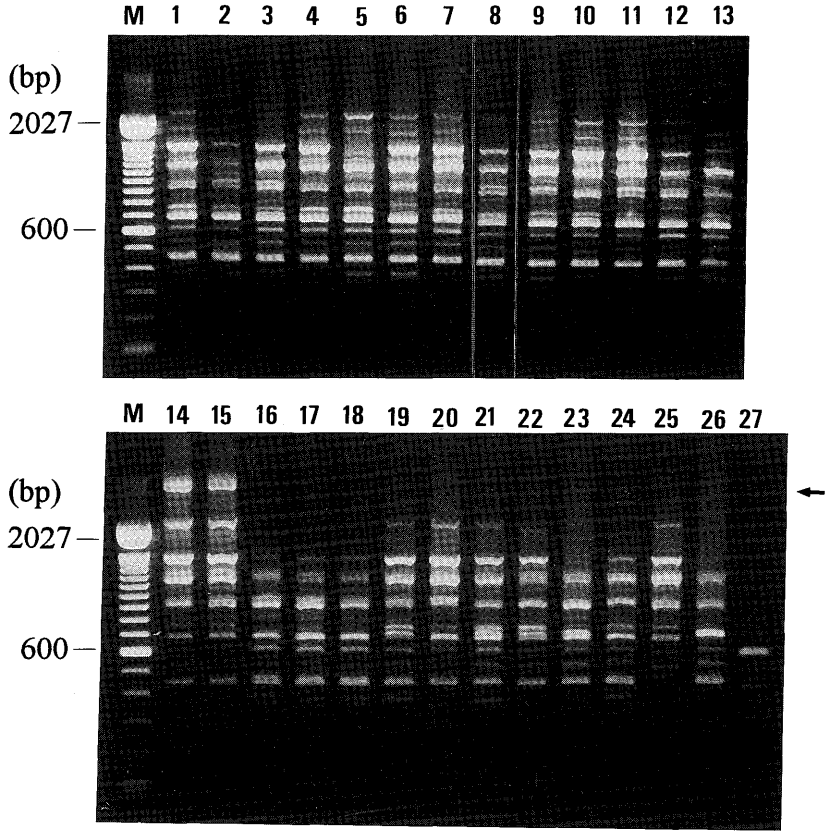

Fig. 3. RAPD profiles from 27 accessions of genus *Glycine* subgenus Soja using primer OPB–05. M: DNA marker; Acc. no.: *G. max* (1–13), *G. gracilis* (14–15), *G. formosana* (16–18), *G. soja* (19–27).

been reported that *G. soja* (*G. formosana*) from northern Taiwan and *G. max* had the similarity coefficients between 0.615 to 0.653 by RFLP analysis. Moreover, it is not as close to *G. max* as *G. soja* is. As a result, it can be separated as a group of its own. From the above reasons, we can consider subgenus Soja as three species, *G. formosana*, *G. soja,* and *G. max*.

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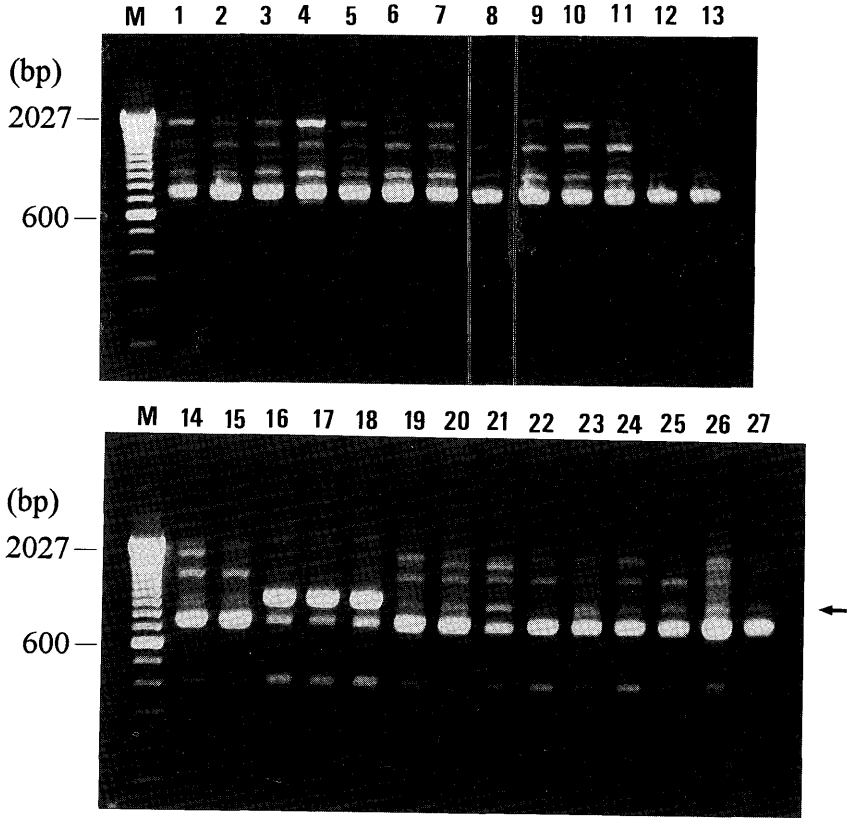

Fig. 4. RAPD profiles from 27 accessions of genus *Glycine* subgenus Soja using primer OPB–11. M: DNA marker; Acc. no.: *G. max* (1–13), *G. gracilis* (14–15), *G. formosana* (16–18), *G. soja* (19–27).

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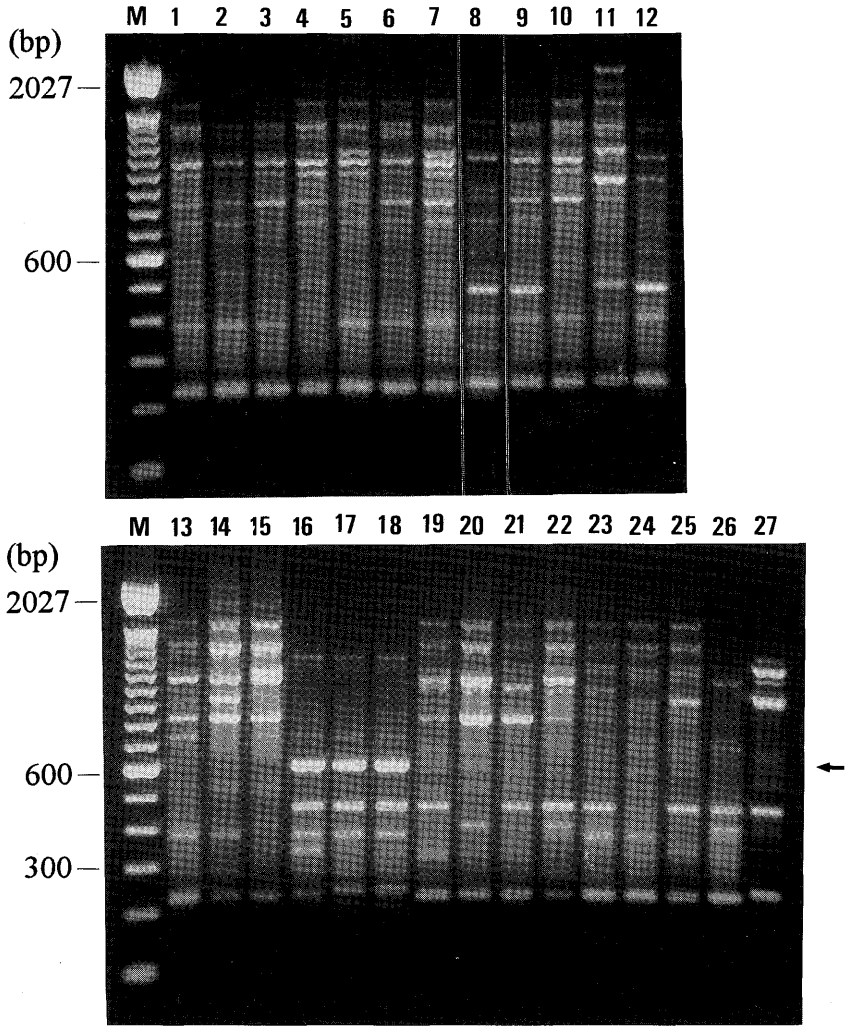

Fig. 5. RAPD profiles from 27 accessions of genus *Glycine* subgenus Soja using primer OPB–12. M: DNA marker; Acc. no.: *G. max* (1–13), *G. gracilis* (14–15), *G. formosana* (16–18), *G. soja* (19–27).

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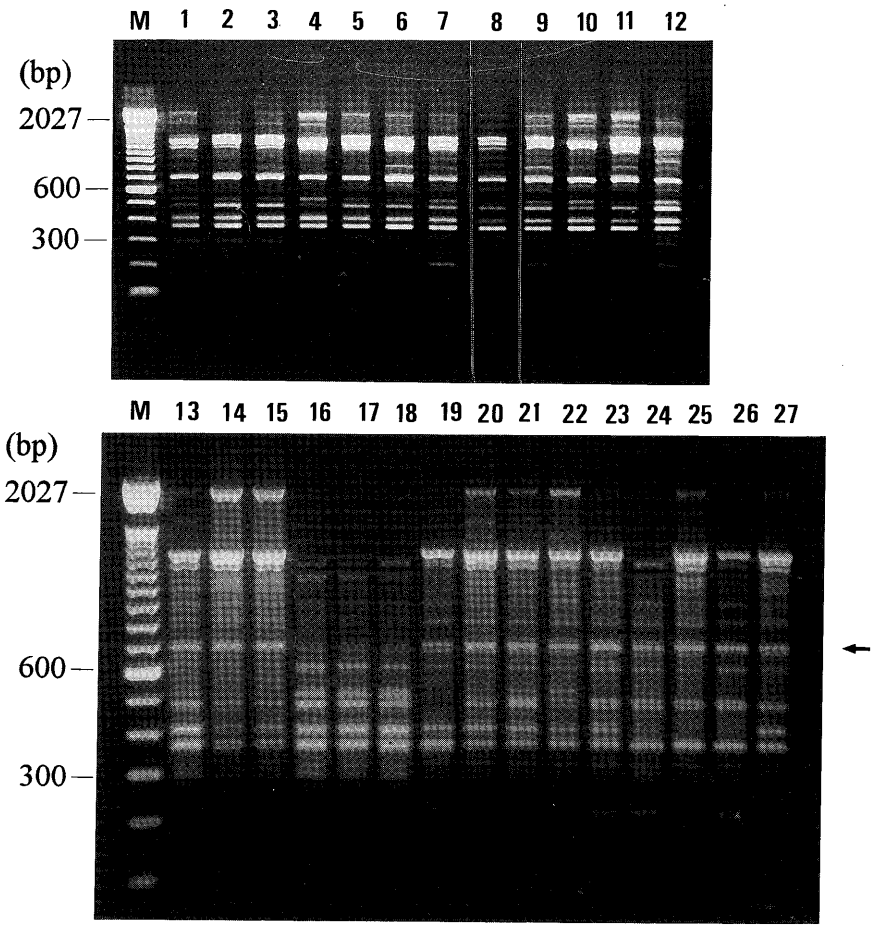

Fig. 6. RAPD profiles from 27 accessions of genus *Glycine* subgenus Soja using primer OPA–15. M: DNA marker; Acc. no.: *G. max* (1–13), *G. gracilis* (14–15), *G. formosana* (16–18), *G. soja* (19–27).

曾　富生, 林　子凱, 呉　詩都：**DNA 多型による台湾産ダイズ属植物の遺伝的変異の解析**

台湾のツルマメはホソバツルマメと呼ばれ，その学名は Hosokawa（1932）によって *Glycine formosana* とされた．しかし，Hermann（1962）及び Chuang and Huang（1966）は，日本や中国などのツルマメと同種の *G. ussuriensis*（*G. soja*）とし，Huang and Ohashi（1977）により *G. soja*，及び Huang and Huang（1987）により *G. max* ssp. *soja* と記載されたこともある．最近 Tateishi and Ohashi（1992）は，この植物の形質変異を検討した結果より，*G. max* ssp. *formosana*（Hosok.）Tateishi & H.Ohashi（1992）と命名した．そこで，本研究においては，ホソバツルマメ及び Soja 亜属に属する *G. soja*, *G. gracilis*, *G. max* について，random amplified polymorphic DNA（RAPD）を用いて系統関係を解析した．RAPD 分析の結果，4 種の中で最も近縁な種は *G. gracilis* と *G. max* で，最も系統的に離れている種は *G. formosana* だった．またこの 4 種は A）*G. max*, *G. gracilis*; B）*G. soja*; C）*G. formosana* の 3 グループに分かれた．この結果より，ホソバツルマメの学名には *G. formosana* を使用するのが良いと考えられた．

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